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Original article

Increased production of natural moisturizing factors and bleomycin hydrolase activity in elderly human skin

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ABSTRACT

Background: Bleomycin hydrolase (BH), which is expressed in the stratum granulosum and lower stratum corneum (SC), is involved in final filaggrin degradation. Furthermore, BH plays an essential role in producing free amino acids, which constitute the majority of natural moisturizing factors (NMF). However, the effects of BH expression and protease activity on human skin aging remain unclear.
Objective: This study was designed to evaluate the activity and expression patterns of BH in SC extracts from healthy young and elderly individuals.
Methods: SC samples were collected by tape stripping. BH activity was assessed by measuring the citrulline aminopeptidase activity. BH expression was determined by Western blotting, and NMF was quantified by liquid chromatography/mass spectrometry. Skin barrier function was determined by measuring SC hydration, transepidermal water loss (TEWL), and skin pH.
Results: The activity and expression of BH were higher in the elderly skin than in young skin, and BH activity was correlated with BH expression levels. Evaluation of the NMF showed that the levels of total amino acids, such as glycine, serine, aspartic acid, citrulline, pyrrolidone carboxylic acid (a metabolite of glutamic acid), and trans-urocanic acid (a metabolite of histidine), were significantly higher in elderly skin than in young skin. Moreover, SC hydration and TEWL were significantly lower in elderly, indicating dry skin, and pH was significantly higher in elderly, indicating greater skin alkalinization.
Conclusion: These results suggest that BH activity and expression, as well as NMF amino acids, increase in elderly people as compensatory mechanisms against dry skin.

1. Introduction

The natural moisturizing factor (NMF) in the stratum corneum (SC) is a combination of water-soluble components that are highly hygroscopic and play an essential role in maintaining the flexibility of the SC [1–3]. NMF consists primarily of amino acids (40 %) and pyrrolidone carboxylic acid (PCA, 12 %), which are derived from deiminated filaggrin molecules [2,3]. The remaining components of NMF in the SC are lactate, sugars, and urea [2,3]. These hygroscopic molecules act as efficient humectants in the corneocytes. NMF compounds represent roughly 20–30 % of the dry weight of the SC [2,3].

Profilaggrin is a highly phosphorylated protein of approximately 500 kDa that localizes to the keratohyalin granules of the stratum granulosum (SG) [4,5]. Profilaggrin is dephosphorylated and cleaved into 10–12 filaggrin monomers, each approximately 35 kDa in size, during the terminal differentiation of the epidermis [4,5]. Filaggrin monomers attach to the keratin fibers of keratinocytes, leading to the formation of corneocytes [4]. Additionally, filaggrin monomers are deiminated by peptidylarginine deiminase to generate citrulline residues [4,6,7]. Deiminated filaggrin is completely degraded into free amino acids by enzymes such as caspase-14, calpain-1, and bleomycin hydrolase (BH) [1,6]. BH is a key enzyme in the final step of filaggrin degradation to produce NMF amino acids [8].

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BH is a neutral cysteine protease widely expressed in mammalian tissues that hydrolyzes and inactivates bleomycin, an antitumor antibiotic [9]. In particular, newborn BH knockout mice display a transient ichthyosis-like phenotype that markedly resembles the phenotype of filaggrin-deficient flaky tail *ft/ft* mice [9–11]. Although BH has broad aminopeptidase activity, it is most effective in cleaving citrulline residues [8]. BH is localized to the SG and lower SC of the healthy epidermis [8,12], but its expression is decreased in the lesional skin of patients with atopic dermatitis, psoriasis, and dry skin [1,12,13]. In addition, BH activity in healthy children is higher in winter than in summer [14].

It remains unclear, however, whether BH expression and citrulline aminopeptidase activity differ in the skin of healthy young and elderly participants. Therefore, the present study evaluated BH expression and activity, as well as NMF contents, of SC extracts obtained by tape stripping of the lumbar skin from healthy young and elderly individuals.

2. Materials and methods

2.1. Reagents

Methanol (HPLC grade > 99.8 %), chloroform (HPLC grade > 99.7 %), acetonitrile (HPLC grade > 99.8 %), and lactic acid (guaranteed reagent) were purchased from Kanto Chemical (Tokyo, Japan). Ultrapure water, formic acid, and ammonium formate were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan), as well as standard solutions consisting of mixtures of type H amino acids and L-asparagine, and mixtures of L-tryptophan, L-glutamine, L-citrulline and L-ornithine monohydrochloride. DL-pyroglutamic acid and trans-urocanic acid (trans-UA) were purchased from Tokyo Chemical Industry (Tokyo, Japan). L-methionine sulfone was purchased from Sigma (St Louis, MO, USA).

2.2. Study design

This study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Medical Ethical Committee of Juntendo University Urayasu Hospital (Approval No: U13–0003-U01). Written informed consent was obtained from all study participants. The study participants comprised 27 volunteers aged 20–39 years, classified as young, and 33 volunteers over 60 years old, classified as elderly. None of the volunteers had a history of skin disease or other physical disorders with cutaneous manifestations.

Experiment 1: Four SC samples were collected at the same site by tape stripping from the lumbar skin of 13 young (7 males, 6 females, mean age of 30.7 ± 4.9 years) and 15 elderly (8 males, 7 females, mean age of 74.1 ± 8.5 years) participants. The samples were specified as the first, second, third, and fourth SC tapes, sequentially, in which BH activity and expression were measured. SC hydration, transepidermal water loss (TEWL), and skin pH were also analyzed.

Experiment 2: SC samples were collected from the lumbar skin of 14 young (4 males, 10 females, mean age of 28.2 ± 5.0 years) and 18

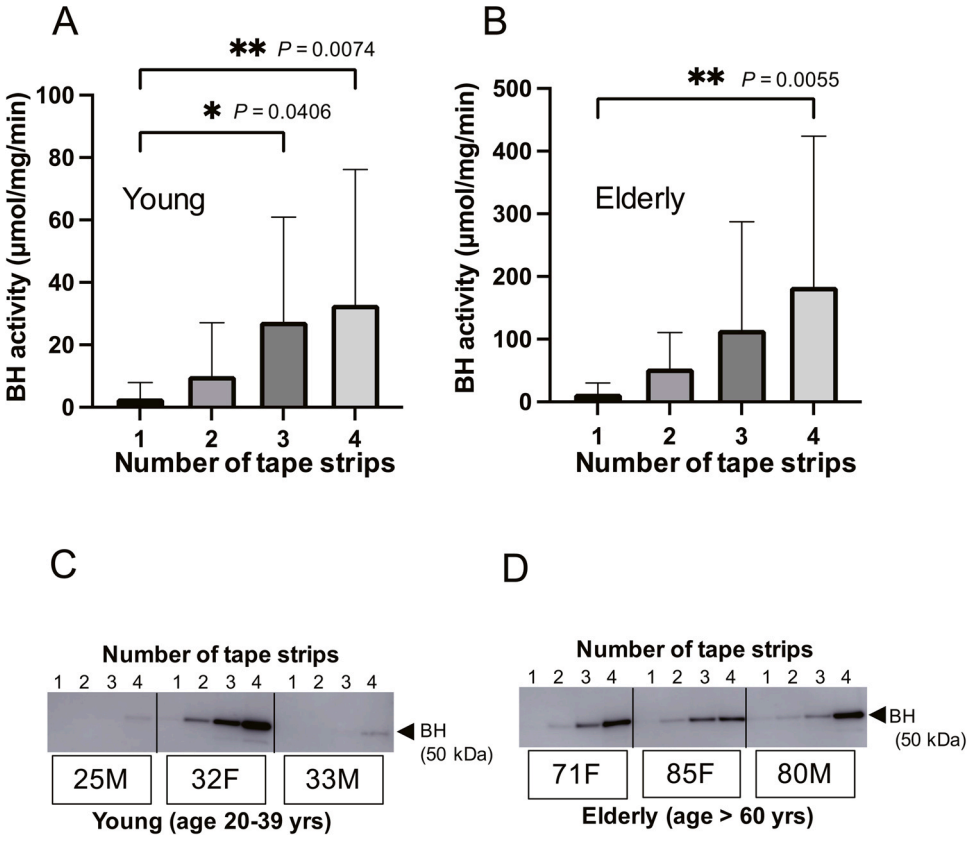

Fig. 1. Detection of BH activity and expression in SC of young and elderly skin. (A and B) Levels of citrulline aminopeptidase activity, an indicator of BH activity, in SC extracts of 13 young (A) and 15 elderly (B) participants. The horizontal axes indicate the number of tape strips performed at the same site. $^{*}P < 0.05$, $^{**}P < 0.01$ by two-way ANOVA with Bonferroni's multiple comparison tests. (C and D) Measurements of BH expression by Western blotting in SC extracts of three young (C) and three elderly (D) participants (yrs: years old, M: male, and F: female). Each sample contained 0.6 μg of total protein extract.

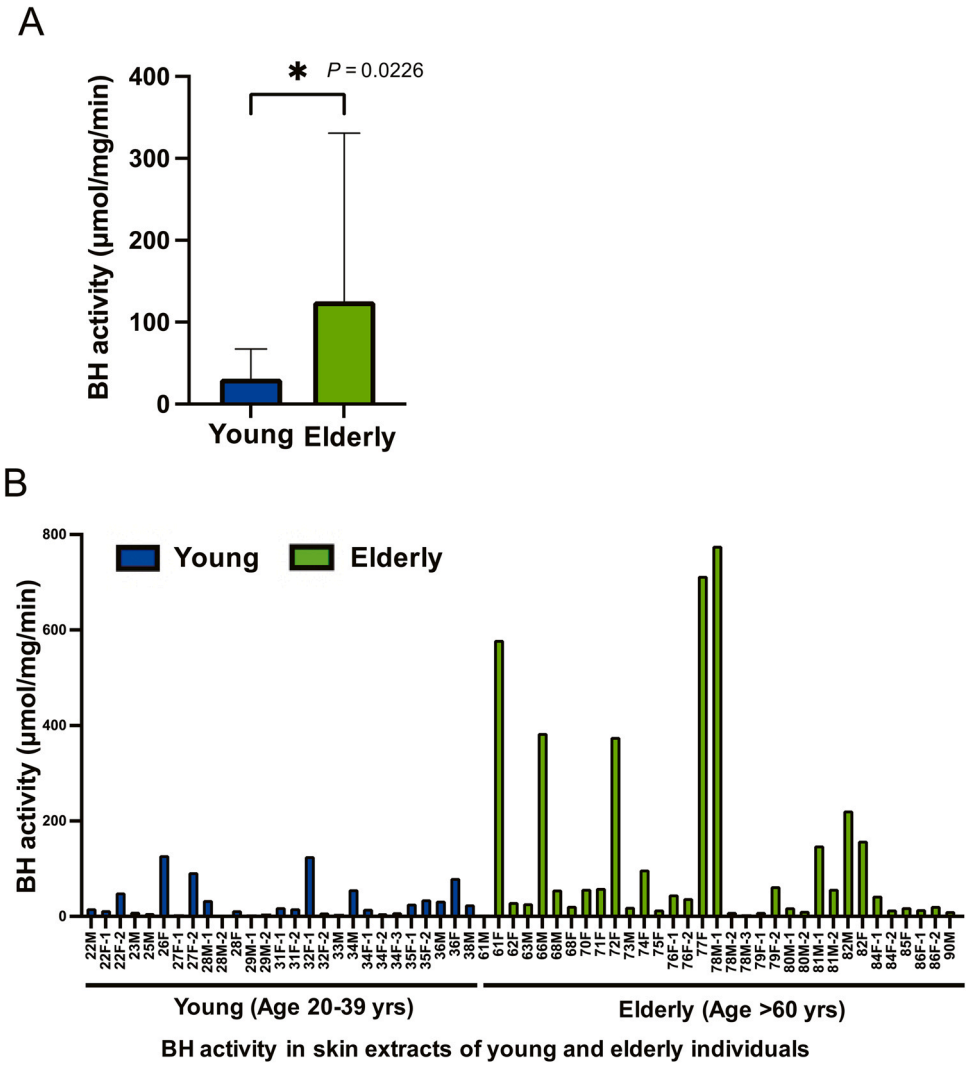

Fig. 2. BH activity in skin extracts of young and elderly participants. (A) BH activity in the fourth SC extracts of 27 young and 33 elderly participants. *$P = 0.0226$ using Student's t-tests. (B) BH activity in skin extracts of young and elderly individuals. Age (years old) and gender (M: male and F: female). Different individuals of the same age and gender are included.

elderly (7 males, 11 females, mean age of 76.3 ± 8.1 years) participants, as described above. The first, second, and third SC samples were mixed. Then, NMF was quantified and BH activity was measured in these combined SC samples. BH activity and expression were analyzed in the fourth SC samples. SC hydration, TEWL, and skin pH were also analyzed.

In Experiment 1, we analyzed the BH activity and expression as a preliminary experiment. The amount of SC extracts was insufficient to quantify the NMF contents, therefore we added new samples in Experiment 2. In Experiments 1 and 2, because BH was mainly expressed in the lower SC and SG, we analyzed BH activity and expression in the fourth SC samples which stably detected BH. All samples were collected in the autumn.

2.3. Collection and extraction of SC protein

To collect corneocytes from the lumbar skin, cellophane tape (Nichiban, Tokyo, Japan) was attached and repeatedly pressed 20 times by hand over the entire area. The same procedure was performed four times at each site. After removing the tape, a 16 cm^2 (2 × 8 cm) section was cut into small pieces and immersed in 1 mL of extraction buffer (0.1 M Tris-HCl, pH 8.0, 0.15 M NaCl, 0.1 % Tween 20). The samples were sonicated three times for 20 s, and the SC extract was obtained by centrifugation. The total protein concentrations were measured using BCA Protein Assay Kits (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

2.4. Measurement of BH activity in SC

BH activity (citrulline aminopeptidase activity) in the extracts was measured by incubating each extract with 0.1 mM citrulline 4-methylcoumaryl-7-amide (BACHEM, Bubendorf, Switzerland) in 0.1 mM Tris-HCl (pH 7.5) containing 10 mM dithiothreitol and 5 mM ethylenediaminetetraacetic acid for 30 min at 37 °C. Fluorescence intensity was measured at 355 and 460 nm using an ARVO-X4 Multiplate Reader (Perkin Elmer, Waltham, MA, USA).

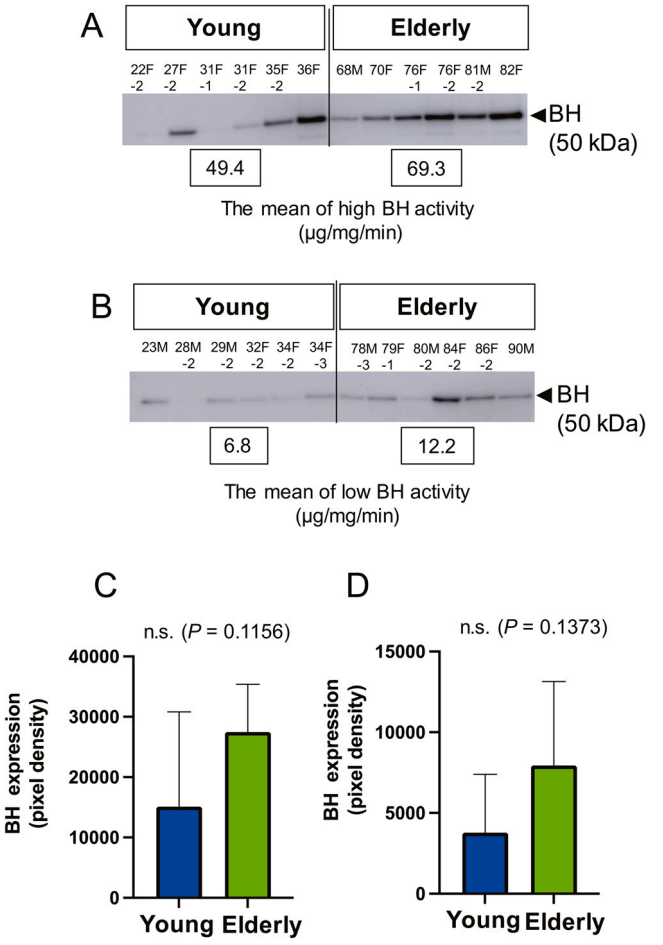

Fig. 3. BH expression in SC of young and elderly participants. Western blotting of BH expression in fourth SC extracts of younger participants with low and high BH activity (A) and elderly participants with low and high BH activity (B). Western blotting of BH expression in the fourth SC extracts of six young and six elderly participants with (A) high BH activity (mean of BH activity, young: 49.4, elderly: 69.3 μg/mg/min), or with (B) low BH activity (mean of BH activity, young: 6.8, elderly: 12.2 μg/mg/min). Each sample contained 0.6 μg of total protein extract. Semi-quantitative analyses of the bands with high BH (C) and low BH (D) activity using Image J software. Data were evaluated using Student's t-tests.

2.5. Western Blot analysis

SC tape-stripped extracts were selected from the six elderly and six young subjects with the highest or lowest BH activity in Experiment 2, respectively. The reason being there is a limit on the number of sample lanes in Western blot, and semi-quantification of Western blot image was needed to analyze the same membrane. SC extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10 % polyacrylamide gels (e-PAGEL E-T10L, ATTO, Tokyo, Japan) and then transferred to polyvinylidene fluoride membranes (Millipore, Danvers, MA, USA). The membranes were blocked with 2 % bovine serum albumin and incubated overnight at 4 °C with rabbit polyclonal anti-BH IgG antibody (1:1000 dilution, 14941-1-AP, Proteintech, Rosemont, IL, USA). The membranes were washed with Tris-buffered saline containing Tween 20 (TBST) and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG superclonal secondary antibody (1:10000 dilution, Thermo Fisher Scientific). After washing with TBST, the bands were visualized using a SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) and semi-quantitatively analyzed using Image J software (National Institute of Health, Bethesda, MD, USA).

2.6. Quantitative analysis of NMF in SC

Each collection of first, second, and third SC tape (1.5 × 5 cm, total of three sheets combined) was immersed in 4.0 mL of 90 % methanol containing 1 μM methionine sulfone and sonicated for 10 min. Then, the solution was removed, and the tape was immersed in 2.0 mL of 90 % methanol and sonicated for 10 min. The solution was removed and combined with the first solution, and the samples were dried under a stream of nitrogen. A 500-μL aliquot of ultrapure water and 500 μL of chloroform were added to stoppered test tubes. Following liquid-liquid partition and centrifugation, 50 μL of the upper aqueous layer was combined with 150 μL of ultrapure water and subjected to liquid chromatography/mass spectrometry (LC/MS) analysis.

NMF in the SC was quantified using an Agilent 6125B Series Single Quadrupole LC/MS (Agilent Technologies Co., Santa Clara, CA, USA) with an Intrada Amino Acid column (3 × 100 mm, 3-μm particle size, Imtakt, Kyoto, Japan). A binary gradient was performed with mobile phase A of acetonitrile/formic acid (v/v, 100/0.1) and mobile phase B of water containing 100 mM ammonium formate. The elution protocol consisted of 14 % B from 0 to 6 min, 14–33 % B from 6 to 13 min, 33–62 % B from 13 to 15 min, 62–100 % B from 15 to 21 min, 100 % B from 21 to 26 min, 14–100 % B from 26 to 27 min, and 14 % B from 27 to 35 min. The flow rate was 0.6 mL/min, the column temperature was 35 °C, and the injection volume was 5 μL. The mass spectrometry parameters were established as follows: a flow of heated dry nitrogen gas, 10.0 L/min; nebulizer gas pressure, 50 PSIG; heater temperature of nitrogen gas, 350 °C; capillary voltage, 2500 V; and fragmenter voltage, 80 V. Each amino acid, as well as PCA and UA, was detected by selected ion monitoring of *m/z* [M+H]+ . Lactic acid was detected by selected ion monitoring of *m/z* [M-H]-.

2.7. Assessment of skin barrier function

TEWL, SC hydration, and skin pH were evaluated using a VapoMeter, Moisture MeterSC (Delfin Technologies Ltd.), and LAQUA act (HORIBA, Kyoto, Japan), respectively. The evaluations were performed at SC tape-stripping sites, according to the manufacturers' instructions.

2.8. Statistical analysis

Data are expressed as mean ± SD and compared using Student's unpaired t-tests or by two-way analysis of variance (ANOVA) with Bonferroni's multiple comparison tests, as appropriate. Correlations were evaluated using Pearson correlation coefficient. All statistical analyses were performed using Prism 8 software (Graphpad Software Inc, San Diego, CA, USA.), and differences with $P < 0.05$ were considered significant.

3. Results

3.1. Detection of BH activity and expression in SC tape extracts

BH activity gradually increased from the first to the fourth SC tape extracts in young and elderly participants (Fig. 1A and B). Regardless of age, BH expression also gradually increased with each additional tape strips (Fig. 1C and D).

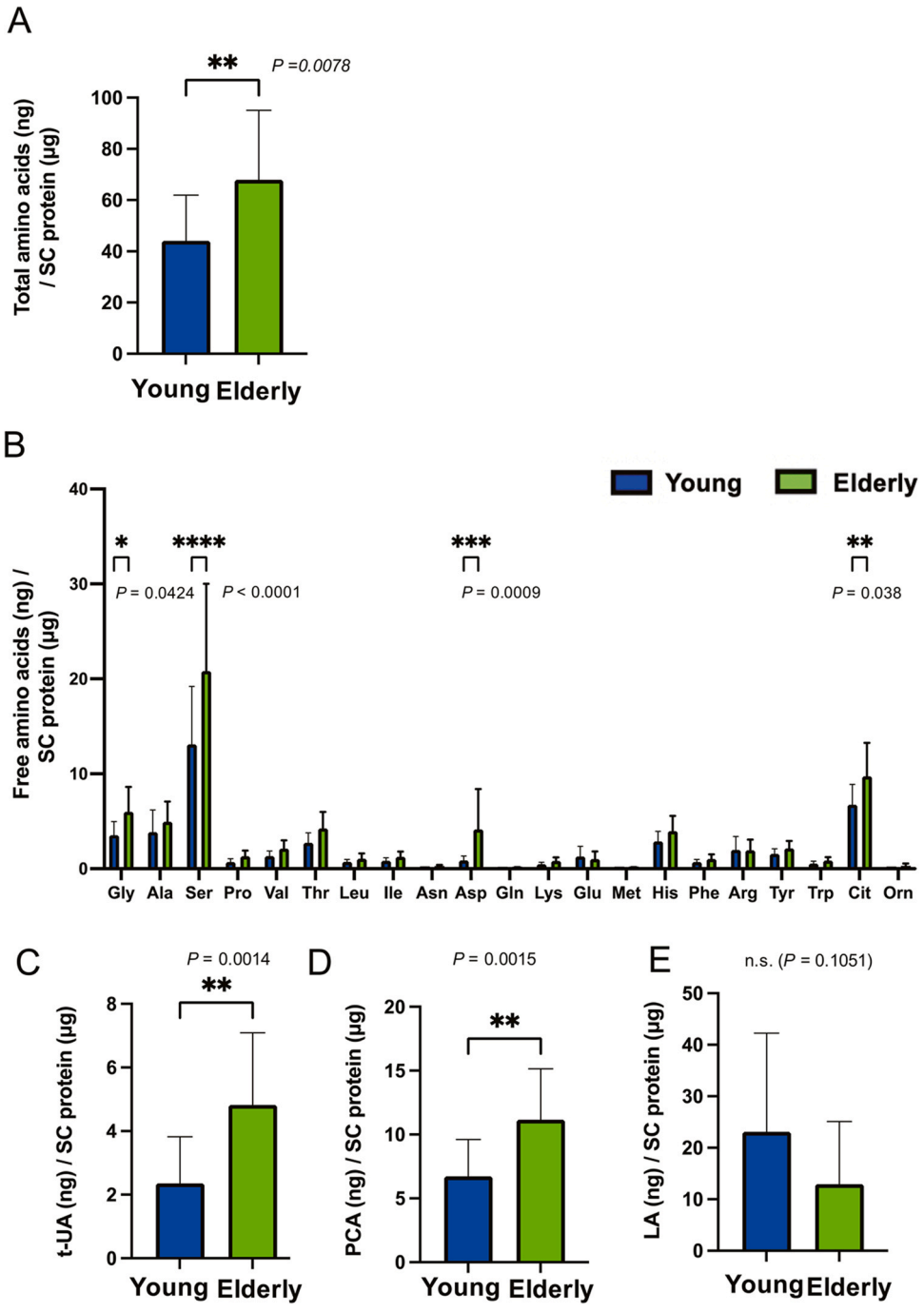

Fig. 4. Quantification of NMF in SC of young and elderly participants. NMF in the mixed tapes of first, second, and third SC extracts of 14 young and 18 elderly participants. (A) Total amino acid contents (ng) in SC protein (μg). $^{****}P = 0.0078$ using Student's t-tests. (B) Mean content of each amino acid (ng) in SC protein (μg) from individual young and elderly participants. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ by two-way ANOVA with Bonferroni's multiple comparison tests. (C–E) Mean content (ng) of (C) pyrrolidone carboxylic acid (PCA), (D) trans-urocanic acid (UA) and (E) lactate in SC protein (μg). Data were evaluated using Student's t-tests (PCA: $^{**}P = 0.0015$, trans-UA: $^{**}P = 0.0014$).

3.2. Comparison of BH activity and expression in SC tape extracts among young and elderly participants

BH activity and expression were compared among SC tape extracts of young and elderly participants. Because BH is mainly expressed in the SG and lower SC, its expression was analyzed in the fourth SC tape extracts. BH activity was significantly higher in the fourth SC extracts from the elderly than from young participants ($P = 0.0226$) (Fig. 2A and B). Similar results were observed in the combined extracts of the first, second, and third SC samples (Fig. S1). BH expression in the fourth SC extracts of young and elderly was also compared by Western blotting (Fig. 3A and B). Evaluation of SC extracts from younger participants with high and low BH activity and elderly participants with low and high BH activity, considering six

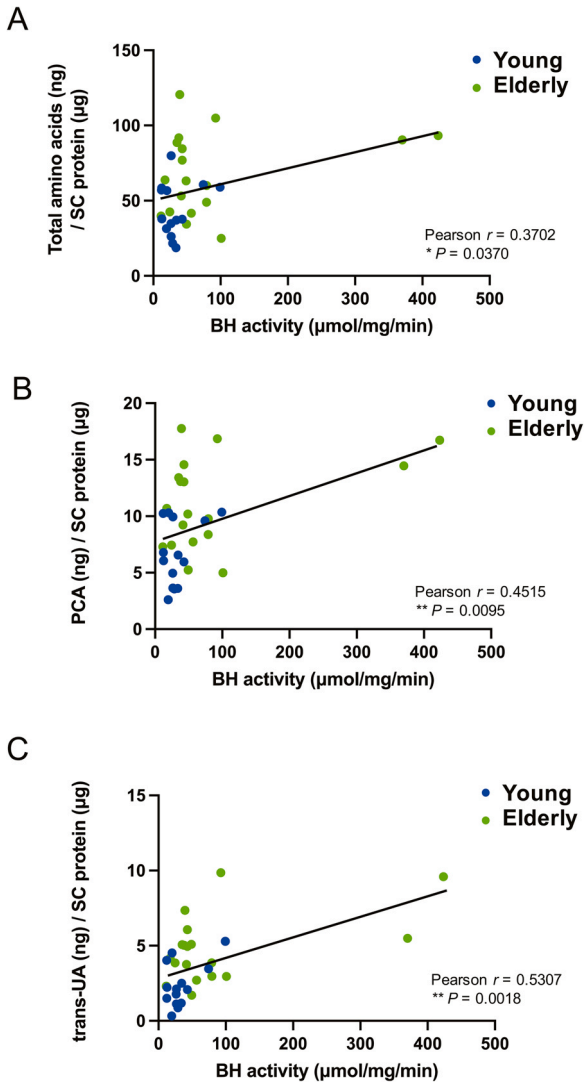

Fig. 5. Correlation analysis among BH activity, total amino acids, PCA, and trans-UA in young and elderly participants. Scatterplots of correlations among BH activity, total amino acid contents (A), PCA (B), and trans-UA (C) in mixed tapes of first, second, and third SC extracts of young and elderly skin. Correlation coefficients (r) were determined by Pearson correlation tests. $P < 0.05$ was considered significant.

individuals in each group, showed that BH expression tended to be higher in samples from the elderly than from young participants (Fig. 3C and D). Moreover, BH activity was correlated with BH expression levels ($r = 0.6962$, $P = 0.0002$) (Fig. S2).

3.3. Quantification of amino acids and their derivatives as NMFs in SC extracts of young and elderly skin

BH plays an essential role in the production of NMF amino acids. Thus, free amino acids and their derivatives were quantified in SC extracts from young and elderly participants. BH activity in the fourth SC tape extracts was correlated with its activity in the mixtures of first, second, and third SC tape extracts ($r = 0.8429$, $P < 0.0001$) (Fig. S3). Determination of NMF levels in the mixtures of first, second, and third SC tape extracts in young and elderly participants showed that the levels of total amino acids were significantly higher in the elderly than in young participants ($P = 0.0078$) (Fig. 4A). In particular, the levels of glycine ($P = 0.0424$), serine ($P < 0.0001$), aspartic acid ($P = 0.0009$), and citrulline ($P = 0.038$) were higher in elderly participants (Fig. 4B), as were the levels of amino acid derivatives, trans-UA ($P = 0.0014$, Fig. 4C) and PCA ($P = 0.0015$, Fig. 4D). However, lactic acid (LA) levels did not significantly differ (Fig. 4E). Additionally, BH activity was correlated with the levels of total amino acids ($r = 0.3702$, $P = 0.0370$) (Fig. 5A), PCA ($r = 0.4515$, $P = 0.0095$) (Fig. 5B), and trans-UA ($r = 0.5307$, $P = 0.0018$) (Fig. 5C).

3.4. Evaluation of skin barrier function

Evaluation of skin barrier function showed that SC hydration ($P < 0.0001$; Fig. 6A) and TEWL ($P = 0.0016$; Fig. 6B) were significantly lower in the skin of the elderly than that of younger participants, whereas skin pH was higher ($P = 0.0480$; Fig. 6C), indicating alkalinization of the skin surface. BH activity did not correlate significantly with SC hydration, TEWL, or skin surface pH (Fig. S4).

4. Discussion

BH is a neutral cysteine protease that plays an essential role in the final step of filaggrin degradation during NMF production [8]. The present study showed that BH expression, BH protease activity, and total amino acid contents in the SC were higher in the elderly than in younger participants.

Filaggrin is a histidine and arginine-rich protein [15]. The present study showed that during terminal differentiation both histidine and glutamic acid, which are partially metabolized to trans-UA and PCA, respectively, [16] were significantly higher in the SC of the elderly than in that of the younger participants. In contrast, arginine residues in filaggrin are partially deiminated to citrulline by peptidylarginine deiminase [6,7]. Deiminated filaggrin is highly susceptible to degradation by BH [8], suggesting that citrulline content is significantly higher in the elderly with high BH activity than in younger subjects. Although NMF amino acids and their derivatives have been reportedly lower in the elderly [17], recent reports have suggested that the levels of filaggrin metabolites, such as free amino acids and their derivatives, are higher in elderly individuals [15,18,19]. The high amounts of NMF amino acids in elderly skin are thought to result from a slower SC turnover [20]. Delayed SC turnover may permit sufficient maturation, as well as degradation of filaggrin of corneocytes, to produce NMF [18]. The present study also showed that LA levels tended to be lower in the elderly than in younger participants. LA, a component of sweat, has been reportedly lower in the NMF of the elderly than that of younger subjects due to decreased sweat gland activity [10]. Similarly, SC hydration has been reported to peak at age 40 and then decline [17].

The present study also showed that SC hydration was significantly lower in the elderly than in younger participants, indicating that aged skin is richer in NMF, but exhibits reduced SC hydration. Sebum and glycerol levels in SC [21,22] and aquaporin-3 levels in the skin [23,24] have been reportedly lower in aged skin. A study reported that elderly people tended to drink less water [25]. Overall, these factors may reduce SC hydration in elderly skin, even under NMF-rich conditions.

TEWL was also significantly lower in the elderly than in young individuals. Reduced TEWL in the elderly may be due to factors such as impaired sweating function and SC thickening associated with decreased epidermal keratinocyte turnover rate [18,20,26–28]. However, the mechanism underlying decreased TEWL in the elderly is unclear [27,29,30], indicating a need for further study. Moreover, we observed that skin pH was higher in elderly participants, which is

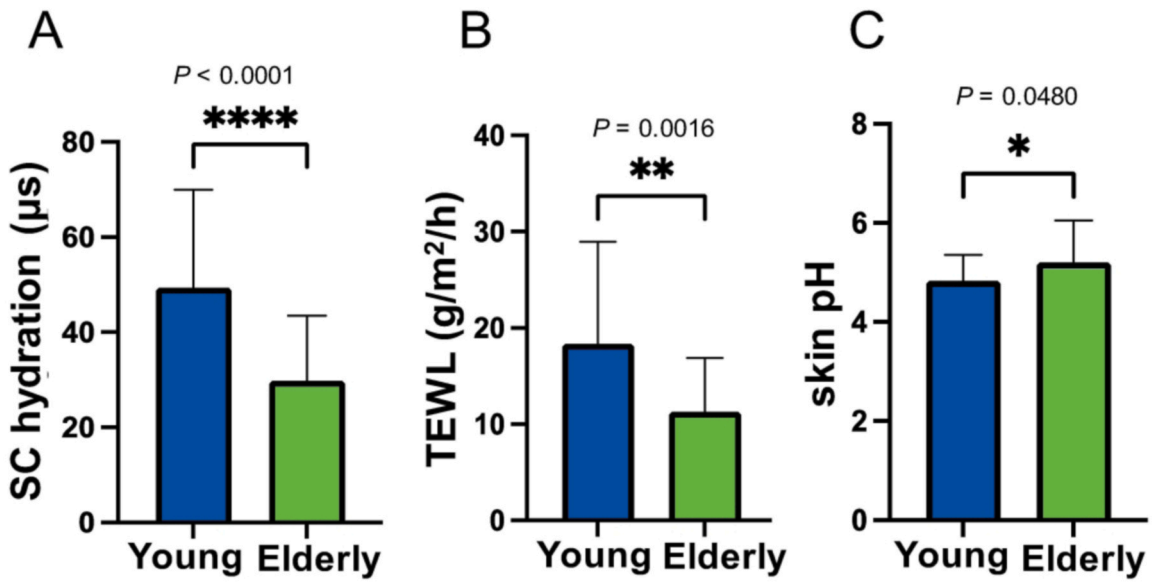

Fig. 6. Analysis of skin barrier function of young and elderly participants. Evaluations of skin barrier functions, including SC hydration, TEWL, and skin pH, at the site of tape stripping in 27 young and 33 elderly participants. Data were evaluated by Student's t-tests (****$P < 0.0001$, **$P < 0.01$ and *$P < 0.05$).

consistent with previous findings [17,31,32]. However, BH activity did not correlate with SC hydration, TEWL, or skin surface pH in the present study, suggesting that alterations of these parameters in elderly skin may involve factors other than BH and NMF amino acids.

In conclusion, the present study showed that BH activity and expression, as well as the NMF amino acid contents, were higher in the skin of the elderly than in younger participants. However, SC hydration was lower in elderly individuals. Taken together, although further research of the mechanism in detail is necessary in the future, these findings suggest that increased BH activity in the elderly is a compensatory mechanism against dry skin.

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CRediT authorship contribution statement

Munehiro Tsurumachi: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft. **Yayoi Kamata:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Validation, Writing – original draft. **Mitsutoshi Tominaga:** Project administration, Investigation, Supervision, Validation, Writing – review & editing. **Junko Ishikawa:** Data curation, Formal analysis, Investigation, Methodology. **Tomoki Hideshima:** Data curation, Formal analysis, Investigation, Methodology. **Eri Shimizu:** Data curation, Formal analysis, Investigation, Methodology. **Takahide Kaneko**: Data curation, Validation. **Yasushi Suga:** Data curation, Validation, Supervision, Writing – review & editing. **Kenji Takamori:** Funding acquisition, Investigation, Supervision, Validation, Writing – review & editing.

Data Availability

The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of interest statement

None of the authors declare any conflicts of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jdermsci.2023.03.001.

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